Comparative gene-expression analysis of periodontal ligament and dental pulp in the human permanent teeth

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감사의 글

박사학위 과정을 시작한 지가 엊그제 같은데 힘들었던 모든 과정을 마치고 논문의 마지막 마무리를 글로 남기려 하니 지난 많은 일들이 스쳐 지나가면서 정말 많은 분들의 도우심이 있었음을 고백하지 않을 수 없습니다. 우선 본 논문의 연구 계획에서부터 완성에 이르기까지 꼼꼼히 지도해 주시고 격려를 아끼지 않으셨던 김성오 지도 교수님께 진심으로 감사를 드립니다. 아울러 관심 있게 지켜봐 주신 손흥규 교수님, 최병재 교수님, 이제호 교수님, 최형준 교수님, 정한성 교수님, 문석준 교수님께도 깊은 감사를 드리며, 실험 설계와 진행에 있어서 아낌없는 관심과 조언, 도움을 주신 송제선 임상교수님께도 감사 드립니다. 또한 실험의 기술적인 부분을 세심하게 도와주신 전미정 조교님을 비롯한 소아치과 의국원들에게도 감사 드립니다.

이 자리에 오기까지 헌신적인 사랑과 한결 같은 정성으로 돌보아 주시며 늘 저의 부족함을 채워주시는 사랑하는 아버님, 어머님과 늘 사랑과 관심으로 인생의 든든한 후원자가 되어주신 사랑하는 장인, 장모님께 우선 깊은 감사를 드립니다. 제가 학위 과정 중 힘들 때 마다 많은 도움을 주신 정회훈 선생님, 한윤범 선생님, 이동우 선생님, 이두영 선생님 외 연세웰키즈치과 모든 가족 여러분들께 감사 드리며, 아울러 항상 옆에서 지켜봐 준 동생 예승이, 매제 오대현 선생님께 감사 드립니다. 마지막으로 어려운 여건 가운데서도 늘 힘이 되어주고 묵묵히 내조해 준 아내와 딸 현서에게 항상 사랑한다는 말을 전하면서 이 논문을 바칩니다.

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Table of contents

List of Figures

List of Tables

Abstract

Comparative gene-expression analysis of periodontal ligament and dental pulp in the human permanent teeth

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(Directed by professor Seong Oh Kim, D.D.S., M.S., Ph.D.)

The periodontal ligament (PDL) and dental pulp tissues of human permanent teeth have a number of differences in their developmental processes, histological characteristics and functions. It can be figured out that these differences are attributable to genetic backgrounds of their cells organized tissues. Meanwhile, most studies were conducted by gene expression analysis using cultured cells of PDL and dental pulp (*in vitro*), but there have been few studies about their gene expression analysis in tissue level (*in vivo*). The purpose of this study was to identify the gene-expression profiles and their molecular biological differences of periodontal ligament and dental pulp tissues from the human permanent teeth. To achieve this, cDNA microarray analysis, quantitative real-time polymerase chain reaction and immunohistochemical stain were used. The cDNA Microarray analysis identified 347 genes with a quadruple or greater difference in expression level between PDL and dental pulp tissues of the permanent teeth, 83 and 264 of which were more plentiful in PDL and dental pulp tissues, respectively. Genes associated with collagen degradation such as those that encode matrix metallopeptidase 3 (*MMP3*), matrix metallopeptidase 9 (*MMP9*), and matrix metallopeptidase 13 (*MMP13*) and with collagen synthesis such as those that encode fibroblast activation protein, alpha (*FAP*) and with bone development and remodeling such as those that encode secreted

phosphoprotein 1 (*SPP1*), bone morphogenetic protein 3 (*BMP3*), acid phosphatase 5, tartrate resistant (*ACP5*), cathepsin K (*CTSK*), and parathyroid hormone-like hormone (*PTHLH*) were more strongly expressed in PDL tissues of permanent teeth. In dental pulp tissues of permanent teeth, genes associated with calcium ion such as those that encode calbindin 1 (*CALB1*), cadherin12, type 2 (*CDH12*), and scinderin (*SCIN*) and with mineralization and formation of enamel or dentin such as those that encode secreted protein acidic and rich in cystein/osteonectin 3 (*SPOCK3*), phosphate regulating endopeptidase homolog, X-linked (*PHEX*), ameloblastin (*AMBN*), and dentin sialophosphoprotein (*DSPP*) were more strongly expressed. The quantitative real-time polymerase chain reaction analysis which was conducted for 6 randomly selected genes was consistent with the results of the cDNA microarray assay. The immunohistochemical staining analysis showed that collagen, type XII, alpha 1 (*COL12A1*), *MMP9*, and *SPP1* was extensively expressed in permanent PDL tissues, but barely expressed in permanent dental pulp tissues. *CALB1* was extensively expressed in the permanent dental pulp tissues, but barely expressed in permanent PDL tissues. These results were also coincided with the cDNA microarray assay data.

Keywords: human permanent teeth, periodontal tissue, dental pulp tissue, cDNA microarray, quantitative real-time polymerase chain reaction, immunohistochemical stain

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I. Introduction

The periodontal ligament (PDL) and dental pulp tissues of human permanent teeth have a number of differences in their developmental processes, histological characteristics and functions. The PDL tissues are components of dental organ that connect tooth to the alveolar bone in the area surrounding the root surfaces. They originate from neural crest derived ectomesenchyme (Miletich and Sharpe 2004), and contain dense connective tissue composed of fibroblasts, Malassez epithelial cell rests, osteoblasts, cementoblasts, vascular cells, and sensory nerve cells, which have the major function of tooth support, regeneration of periodontal tissues, maintaining homeostasis and providing the healing process by periodontal disease or mechanical trauma (Gottlow et al. 1984). In terms of mechanical trauma, ankylosed teeth cannot be moved by orthodontic force because of absence of PDL (Mitchell and West 1975). The mechanical stress loaded onto a tooth is transferred to the PDL and then cells in the PDL respond to the mechanical stress for regulating the absorption and formation of bone matrix by

signaling the surrounding cells (Lekic and McCulloch 1996). The dental pulp, which is originated from dental papilla, is an unmineralized oral tissue composed of soft connective tissue, vascular, lymphatic and nervous elements that occupy the central pulp cavity of dental apparatus. The soft connective tissue composed of fibroblasts, odontoblasts, and undifferentiated mesenchymal cells, which have the characteristics of specific anatomic location and organization being surrounded by hard and inelastic dentin (Abrahao et al. 2006). Several previously performed studies have shown that dental pulp cells have the ability to differentiate into odontoblasts in order to repair the dental damage caused by trauma, caries or dental erosion (Gronthos et al. 2000). For such instances as mentioned, it can be figured out that these differences are attributable to genetic backgrounds of their cells organized tissues.

The 2001 draft sequence of the human genome by the Human Genome Project was unquestionably a great scientific achievement, a turning point for human genetics, and the starting point for human genomics. Three years later, international efforts delivered a high quality finished human genome assembly representing 99% of the euchromatic sequence (Gonzaga-Jauregui et al. 2012). Although the highest quality genome for any organism, it is not sufficient for understanding complicated life phenomena in humans. Therefore, additional information such as functional genomics is required for determining interaction of genes. In recent years, cDNA microarray is one of the powerful tools in functional genomics.

Traditional methods used for mRNA or protein expression, such as reverse transcription polymerase chain reaction (RT-PCR) or Western blot, have the disadvantages of focusing only a few different molecules, taking a long time for their changes, and difficulty in understanding the general picture of changes. cDNA microarray, by contrast, is suitable for large-scale screening of genes or protein expression effectively in the field of dentistry. It enables studying the expression of thousands of genes and proteins simultaneously and the comparison of gene expression level of two different samples. For example, it can offer information on the normal molecular and cellular functions of PDL and pulp tissue and their changes during

disease, and thus enables the search for target protein for dental treatment (Paakkonen et al. 2005).

Most studies were conducted by gene expression analysis using cultured cells of PDL and dental pulp (*in vitro*). Several studies of cultured PDL and dental pulp cells which have used cDNA microarray, such as gene expression in cultured PDL cells under hypoxia by cDNA microarray (Kitase et al. 2009), the cytokines and growth factors expressed by cultured human PDL cells *in vitro* and identification of new genes that are responsive to mechanical deformation (Pinkerton et al. 2008), comparison of dental pulp cell between healthy and caries teeth (McLachlan et al. 2005, Paakkonen et al. 2005), and microarray evaluation of aged-related change in human dental pulp cell (Tranasi et al. 2009), were reported. However, there have been few studies about their gene expression analysis in tissue level (*in vivo*) and direct comparison between PDL and dental pulp cell of human permanent teeth. Therefore, this present study aimed to identify and compare the gene expression profiles and their molecular biological differences of PDL and dental pulp tissues from the human permanent teeth.

II. Materials and Methods

1. PDL and pulp samples

The experimental protocol was approved by the Institutional Review Board of the Yonsei University Dental Hospital, and informed consent to participate was obtained from all of the subjects (#2-2011-0050). The PDL samples were obtained from healthy persons (n=10; from 2 males and 3 females, aged 11-19 years) and the pulp samples were obtained from healthy matured permanent premolar extracted for orthodontic reasons from healthy persons (n=24; from 6 males and 6 females, aged 18-25 years). Extracted teeth were immediately frozen and stored in liquid nitrogen. The PDL tissues were obtained carefully using sterile curettes from the middle-third. Teeth were subsequently crushed with a bolt cutter and the pulp tissues were carefully obtained using a sterile tweezers. And then PDL and pulp tissues immediately submerged in RNA stabilizing reagent (RNAlater, Qiagen, CA, USA).

2. RNA isolation

Total RNA was extracted from PDL or pulp tissues using the RNeasy Fibrous Mini kit® (Qiagen, USA) according to the manufacturer's instructions. The extracted RNA was eluted in 25μl of sterile water. Prior to RNA extraction, tissues were homogenized using a Bullet Blender® Bead (Next Advanced, Inc., NY, USA). RNA concentrations were determined from absorbance values at a wavelength of 260nm using a spectrophotometer (Nanodrop ND-1000®, Thermo Scientific, IL, USA). RNA samples used in this study had 260/280 nm ratios equal to or greater than 1.8.

3. cDNA microarray

Global gene expression analyses were conducted using Affymetrix GeneChip® Human Gene 1.0 ST oligonucleotide arrays (Affymetrix Inc., CA, USA). The sample preparation was performed according to the instructions and recommendations provided by the manufacturer. The average amount of RNA isolated from PDL or pulp tissues was 1μg. RNA quality was assessed by Agilent 2100 bioanalyser using the RNA 6000 Nano Chip® (Agilent Technologies, Amstelveen, The Netherlands), and quantity was determined by NanoDrop ND-1000 (Thermo Scientific).

As recommended by the manufacturer's protocol, 300ng were used. Briefly, 300ng of total RNA from each sample was converted to double-strand cDNA. Using a random hexamer incorporating a T7 promoter, amplified RNA (cRNA) was generated from the double-stranded cDNA template though an IVT (in-vitro transcription) reaction and purified with the Affymetrix sample cleanup module. cDNA was regenerated through a random-primed reverse transcription using adNTP mix containing dUTP. The cDNA was then fragmented by UDG and APE1 restriction endonucleases and end-labeled by terminal transferase reaction incorporating a biotinylated dideoxynucleotide. Fragmented end-labeled cDNA was hybridized to the GeneChip® Human Gene 1.0 ST arrays for 16 hours at 45℃ and 60 rpm as described in the GeneChip Whole Transcript Sense Target Labeling Assay Manual (Affymetrix). After hybridization, the chips were stained and washed in a GeneChip Fluidics Station 450® (Affymetrix) and scanned by using a GeneChip Array scanner 3000 G7® (Affymetrix) and the image data was extracted through Affymetrix Commnad Console software1.1® (Affymetrix). The raw file generated through above procedure meant expression intensity data and was used for the next step. Microarray procedure was done 3 times in each group using 3 samples from each group.

4. Microarray data analysis

Expression data were generated by Affymetrix Expression Console software version1.1® (Affymetrix). For the normalization, RMA (Robust Multi-Average) algorithm implemented in Affymetrix Expression Console software was used. To determine whether genes were differentially expressed between the three groups, a one-way ANOVA was performed on the RMA expression values. A multiple testing correction was applied to the *p*-values of the *F*-statistics to adjust the false discovery rate (Benjamini and Hochberg, 1995). Genes with adjusted *F*-statistic *p*-values <0.05 were extracted. Highly expressed genes in PDL or pulp that showed over 6 or 11 fold differences comparing the signal value of control and each test group, were selected for the further study. In order to classify the co-expression gene group which has similar expression pattern, I performed Hierarchical clustering and K-mean clustering in MEV (MultiExperiment Viewer) software 4.4 (www.tm4.org, Dana-Farber Cancer Institute, MA, USA). The web-based tool, DAVID (the Database for Annotation, Visualization, and Integrated Discovery) was used to perform the biological interpretation of differentially expressed genes. Then, these genes were classified based on the information of gene function in Gene ontology, Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway database (http://david.abcc.ncifcrf.gov/home.jsp).

5. Quantitative RT-PCR

The single-stranded cDNA required in the polymerase chain reaction (PCR) analysis was produced using 500ng of extracted total RNA as a templates for reverse transcription (RT; Superscript III Reverse Transcriptase and random primer, Invitrogen, UK). The RT reaction was performed at 65℃ for 5 minutes, followed by 25℃ for 5 minutes, 50℃ for 1 hour, and 70℃ for 15 minutes to inactivate the activity of the reverse transcriptase. The synthesized cDNA was diluted 10:1 in distilled water and used as a template for quantitative RT-PCR, which was performed using the ABI7300 RT-PCR system (Applied Biosystems, Warrington, UK). Samples of 25μl containing 1x Universal TaqMan Master Mix (4369016, Applied Biosystems), PCR primers at a concentration of 0.9μM and the diluted cDNA were prepared in triplicate. The amplification conditions were 50℃ for 2minutes and 95℃ for 10 minutes followed by 40 cycles of 95℃ for 15 seconds and 60℃ for 1 minute. The following TaqMan gene expression assay primers (Applied Biosystems) were used: ameloblastin (*AMBN*), calbindin 1 (*CALB1*), collagen, type XII, alpha (*COL12A*), dentin sialophosphoprotein (*DSPP*), matrix metallopeptidase 9 (*MMP9*), matrix metallopeptidase 20 (*MMP20*), secreted protein acidic and rich in cystein/osteonectin 3 (*SPOCK3*), secreted phosphoprotein 1 (*SPP1*), and 18S rRNA. ABI 7300 SDS 1.3.1 software (Applied Biosystems) recorded the fluorescence intensity of the reporter and quencher dyes; the results are plotted versus time, quantified as the cycle number. A precise quantification of the initial target was obtained by examining the amplification plots during the early log phase of product accumulation above background [the threshold cycle (Ct) number]. Ct values were subsequently used to determine ΔCt values (ΔCt=Ct of the gene minus Ct of the 18S rRNA control), and differences in Ct values were used to quantify the relative amount of PCR product, expressed as the relative change by applying the equation $2^{-\Delta Ct}$. The specific primer assay ID and product sizes for each gene are listed in Table 1.

Table 1. Quantitative RT-PCR primers used in this study

Genes	Primer Assay ID	Product Size (bp)
AMBN	Hs00212970_m1	61
CALB1	Hs00191821_m1	90
COL12A	Hs00189184_m1	63
DSPP	Hs00171962_m1	67
MMP9	Hs00234579_m1	54
MMP20	Hs01573770_m1	79
SPOCK3	Hs01553242_m1	88
SPP1	Hs00959010_m1	84
18S rRNA	Hs03003631_g1	69

6. Immunohistochemical Staining

In preparation for immunohistochemical (IHC) staining, permanent teeth were fixed in 10% buffered formalin (Sigma, MO, USA) for 1 day, decalcified with 10% EDTA (pH 7.4; Fisher Scientific, TX, USA) for 8 weeks, embedded in paraffin, and sectioned at a thickness of 3μm. Specimens were subjected to IHC staining with antihuman CALB1 (rabbit polyclonal, diluted 1:400; Ab25085, Abcam, Cambrige, UK), MMP9 (rabbit polyclonal, diluted 1:800; Ab38898, Abcam), SPP1 (osteopontin, rabbit polyclonal, diluted 1:800; Ab8448, Abcam), and COL12A1 (rabbit polyclonal, diluted 1:800; Sc-68862, Santa Cruz Biotechnology, Inc., CA, USA). Endogenous peroxidase activity was quenched by the addition of 3% hydrogen peroxide. Sections were incubated in 5% bovine serum albumin to block nonspecific binding. The primary antibodies were diluted to give optimal staining and the sections were incubated overnight. After incubation, EnVision+ System-HRP Labelled Polymer Anti-rabbit (K4003, Dako North America Inc., CA, USA; ready to use) was applied for 20 min. Color development was performed using labeled streptavidin biotin kits (Dako) according to the manufacturer's instructions. The sections were counterstained with Gill's hematoxylin (Sigma). Control sections were treated in the same manner but without treatment with primary antibodies.

III. Results

1. Gene-expression profiles of PDL and dental pulp tissue

Complementary DNA microarray technology was used to compare multiple gene expression profiles representative of PDL and dental pulp tissues in the human permanent teeth. The total data distribution and frequency were confirmed by density and box plots (Figure 1), M-A plots (Figure 2) of the standardized log intensity ratio to the average intensity. The results announced that 347 out of 28,869 genes (1.20%) had a changed expression of a quadruple or greater difference between PDL and dental pulp tissues of the permanent teeth. In the PDL tissues, the expressions of 83 genes were fourfold or more than in the dental pulp tissues (Table 2), while in the dental pulp tissues, the expressions of 264 genes were fourfold or more than in those in the PDL tissues (Table 3).

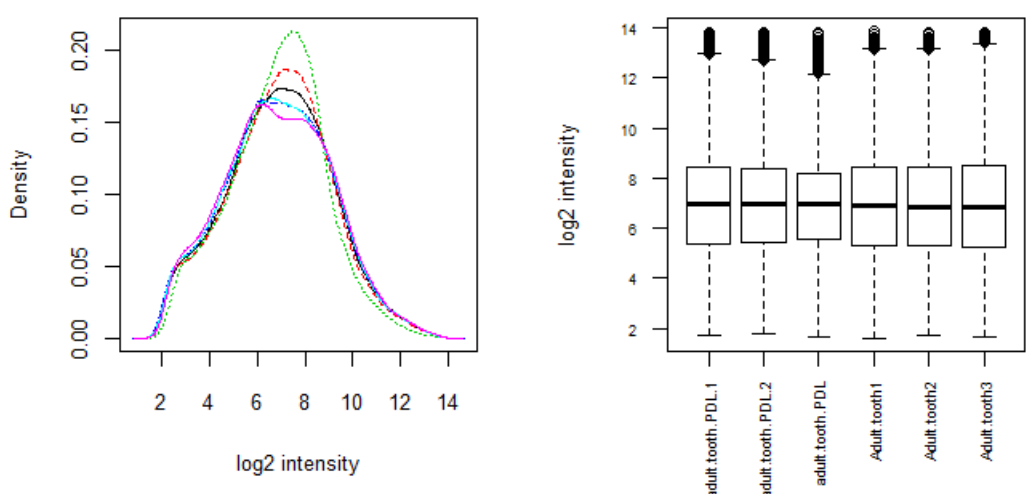

Figure 1. Density plots (left) and box plots (right) for confirmation of total data distribution and frequency

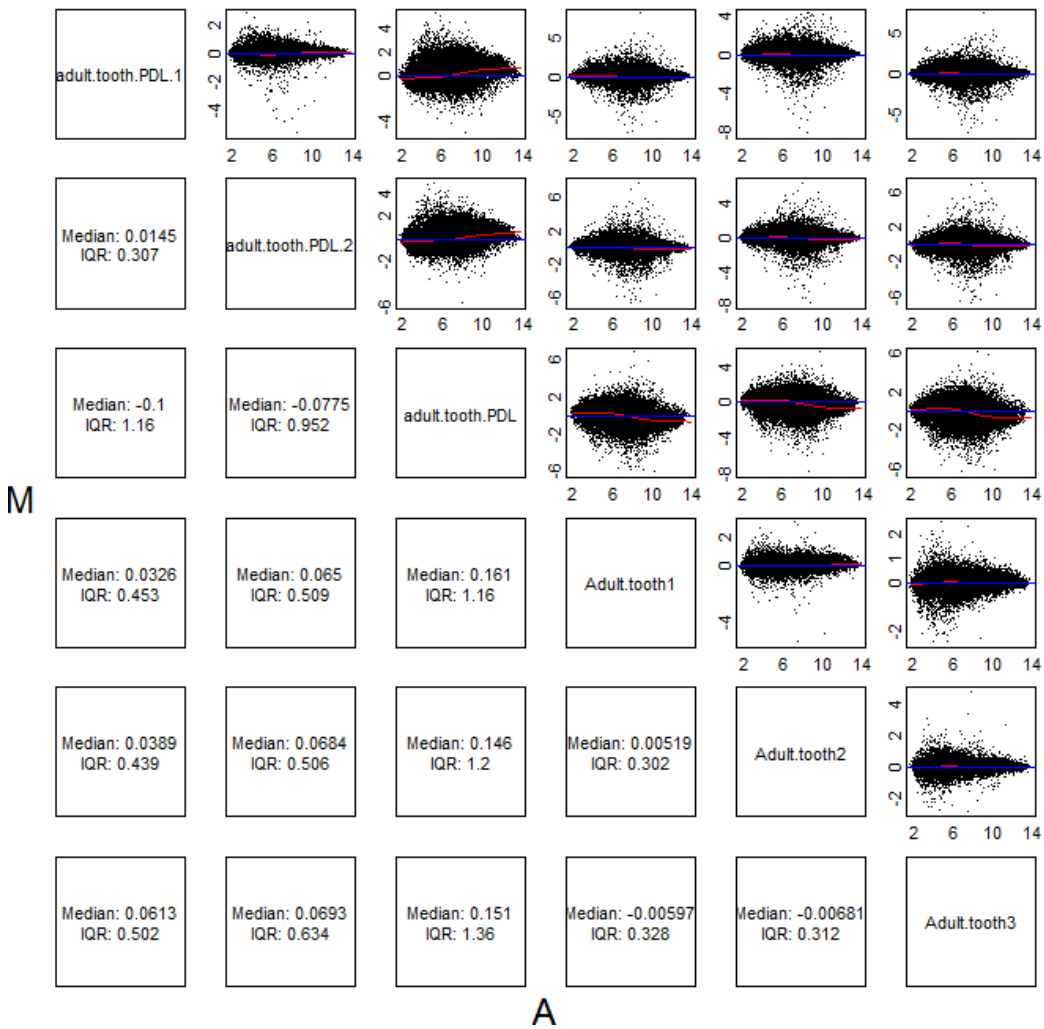

Figure 2. M-A plots

Table 2. Up-regulated genes in the PDL tissue of permanent teeth

Name	Gene Symbol	Fold change	Gene Accession	Cytoband
matrix metallopeptidase 13	*MMP13*	43.56	NM_002427	11q22.3
matrix metallopeptidase 9	*MMP9*	28.53	NM_004994	20q11.2-q13.1
keratin 5	*KRT5*	19.35	NM_000424	12q
collagen, type XII, alpha 1	*COL12A1*	18.20	NM_004370	6q12-q13
immunoglobulin superfamily, member 10	*IGSF10*	16.16	NM_178822	3q25.1
fibroblast activation protein, alpha	*FAP*	15.32	NM_004460	2q23
tenascin N	*TNN*	14.92	NM_022093	1q23-q24
secreted phosphoprotein 1	*SPP1*	13.84	NM_001040058	4q22.1
collagen, type XI , alpha 1	*COL11A1*	12.94	NM_001854	1p21
RAB27B, member RAS oncogene family	*RAB27B*	12.28	NM_004163	18q21.2
plexin C1	*PLXNC1*	11.87	NM_005761	12q23.3
ATPase, H^+ transporting, lysosomal 38kDa, V0 subunit d2	*ATP6V0D2*	11.35	NM_152565	----
bone morphogenetic protein 3	*BMP3*	10.88	NM_001201	4q21
asporin	*ASPN*	10.80	NM_017680	9q22
leucine rich repeat containing 15	*LRRC15*	10.12	NM_001135057	3q29
platelet-derived growth factor receptor-like	*PDGFRL*	8.50	NM_006207	8p22-p21.3
sushi-repeat-containing protein, X-linked 2	*SRPX2*	8.42	NM_014467	Xq21.33-q23
secreted frizzled-related protein 2	*SFRP2*	8.12	NM_003013	4q31.3
acid phosphatase 5, tartrate resistant	*ACP5*	7.85	NM_001111035	19p13.3-p13.2
XG blood group	*XG*	7.66	NM_001141919	Xp22.33
SH3 and cysteine rich domain	*STAC*	7.63	NM_003149	3p22.3
CD109 molecule	*CD109*	7.38	NM_133493	6q13
transmembrane 4L six family member 19	*TM4SF19*	7.36	NM_138461	3q29

Table 2. Up-regulated genes in the PDL tissue of permanent teeth (continued)

Name	Gene Symbol	Fold change	Gene Accession	Cytoband
odontogenic, ameloblast associated	*ODAM*	7.29	NM_017855	4q13.3
angiopoietin-like 2	*ANGPTL2*	7.25	NM_012098	9q34
collagen, type VI, alpha3	*COL6A3*	7.11	NM_004369	2q37
dipeptidyl-peptidase 4	*DPP4*	7.05	NM_001935	2q24.3
retinol binding protein 4, plasma	*RBP4*	6.92	NM_006744	10q23-q24
prune homolog 2	*PRUNE2*	6.88	NM_015225	9q21.2
RAR-related orphan receptor B	*RORB*	6.85	NM_006914	9q22
integrin, alpha 11	*ITGA11*	6.81	NM_001004439	15q23
matrix metallopeptidase 3	*MMP3*	6.75	NM_002422	11q22.3
keratin 14	*KRT14*	6.61	NM_000526	17q12-q21
cathepsin K	*CTSK*	6.60	NM_000396	1q21
fibronectin type III domain containing 1	*FNDC1*	6.41	NM_032532	6q25
prolyl 4-hydroxylase, alpha polypeptide III	*P4HA3*	6.39	NM_182904	11q13.4
sarcoglycan, gamma	*SGCG*	6.11	NM_000231	13q12
corin, serine peptidase	*CORIN*	6.07	NM_006587	4p13-p12
parathyroid hormone-like hormone	*PTHLH*	6.05	NM_198965	12p12.1-p11.2

Table 3. Up-regulated genes in the dental pulp tissue of permanent teeth

Name	Gene Symbol	Fold change	Gene Accession	Cytoband
calbindin 1, 28kDa	*CALB1*	99.53	NM_004929	8q21.3-q22.1
phosphate regulating endopeptidase homolog, X-linked	*PHEX*	68.41	NM_000444	Xp22.2-p22.1
sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 3	*SPOCK3*	67.93	NM_001040159	4q32.3
matrix metallopeptidase 20	*MMP20*	63.35	NM_004771	11q22.3
cadherin 12, type 2 (N-cadherin 2)	*CDH12*	62.88	NM_004061	5p14.3
transferrin	*TF*	55.52	NM_001063	3q22.1
ameloblastin	*AMBN*	48.59	NM_016519	4q21
sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphoring) 3E	*SEMA3E*	45.67	NM_012431	7q21.11
luecine-rich repeat-containing G protein-coupled receptor 5	*LGR5*	42.99	NM_003667	12q22-q23
dentin sialophosphoprotein	*DSPP*	41.24	NM_014208	4q21.3
abhydrolase domain containing 12B	*ABHD12B*	32.82	NM_181533	14q22.1
lipid phosphate phosphatase-repeatd protein type 5	*LPPR5*	26.38	NM_001037317	1p21.3
scinderin	*SCIN*	26.17	NM_001112706	7p21.3
transmembrane protein 156	*TMEM156*	25.26	NM_024943	4p14
serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	*SERPINA3*	24.63	NM_001085	14q32.1
carboxypeptidase A6	*CPA6*	22.05	NM_020361	8q13.2
gamma-aminobutyric acid (GABA) A receptor, beta 1	*GABRB1*	18.98	NM_000812	4p12

Table 3. Up-regulated genes in the dental pulp tissue of permanent teeth (continued)

Name	Gene Symbol	Fold change	Gene Accession	Cytoband
bone morphogenetic protein receptor, type IB	*BMPR1B*	18.19	NM_001203	4q22-q24
ceruloplasmin (ferroxidase)	*CP*	16.92	NM_000096	3q23-q25
ATP-binding cassette, sub-family A (ABC1), member 6	*ABCA6*	16.76	NM_080284	17q24.3
RAN binding protein 3-like	*RANBP3L*	15.99	NM_001161429	5p13.2
potassium channel, subfamily K, member 2	*KCNK2*	15.81	NM_001017425	1q41
G protein-coupled receptor 37 (endothelin receptor type B-like)	*GPR37*	14.46	NM_005302	7q31
protocadherin 20	*PCDH20*	14.37	NM_022843	13q21
ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialytransferase 1	*ST8SIA1*	14.28	NM_003034	12p12.1-p11.2
coxsackie virus and adenovirus receptor	*CXADR*	13.64	NM_001338	21q21.1
solute carrier family 4, sodium bicarbonate cotransporter, member 4	*SLC4A4*	13.15	NM_001098484	4q21
G protein-coupled receptor 63	*GPR63*	12.88	NM_001143957	6q16.1-q16.3
reelin	*RELN*	12.87	NM_005045	7q22
cytoplasmic FMR1 interacting protein 2	*CYFIP2*	12.51	NM_001037332	5q33.3
gastrin-releasing peptide receptor	*GRPR*	12.42	NM_005314	Xp22.2
EPH receptor A5	*EPHA5*	12.20	NM_004439	4q13.1
sodium channel, voltage-gated, type VII, alpha	*SCN7A*	12.02	NM_002976	2q21-q23
cerebellin 2 precursor	*CBLN2*	11.91	NM_182511	18q22.3
WD repeat domain 72	*WDR72*	11.72	NM_182758	15q21.3

2. Gene ontology analysis

To translate the microarray's data to meaningful biologic functional terms and to characterize the groups of functionally related genes, Gene Ontology Consortium (GO) grouping using the DAVID web-based tool was used. These genes were classified based on information regarding gene function in gene ontology from KEGG Pathway database. The GO classes with an *F*-statistic $p<0.05$ following analysis on the basis of their biologic processes and molecular functions are shown in Figure 3, 4 respectively. In broad outlines, the GO classes of the dental pulp tissue are relatively more counted those of the PDL tissue. Especially, there were notable differences of biologic processes in regulation of cell adhesion, neurological system process, signal transduction, and ion transport, and of molecular functions in nucleotide binding, ATP binding, and ion binding.

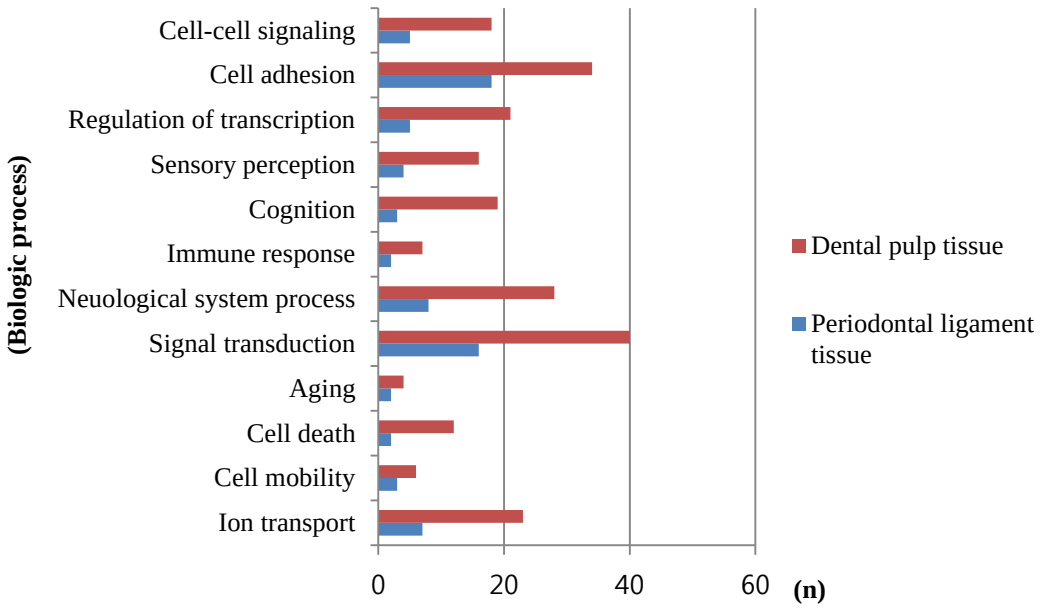

Figure 3. Main categories of genes expressed in the PDL and dental pulp tissues of the human permanent teeth on basis of their biologic processes (*F*-statistic $p<0.05$)

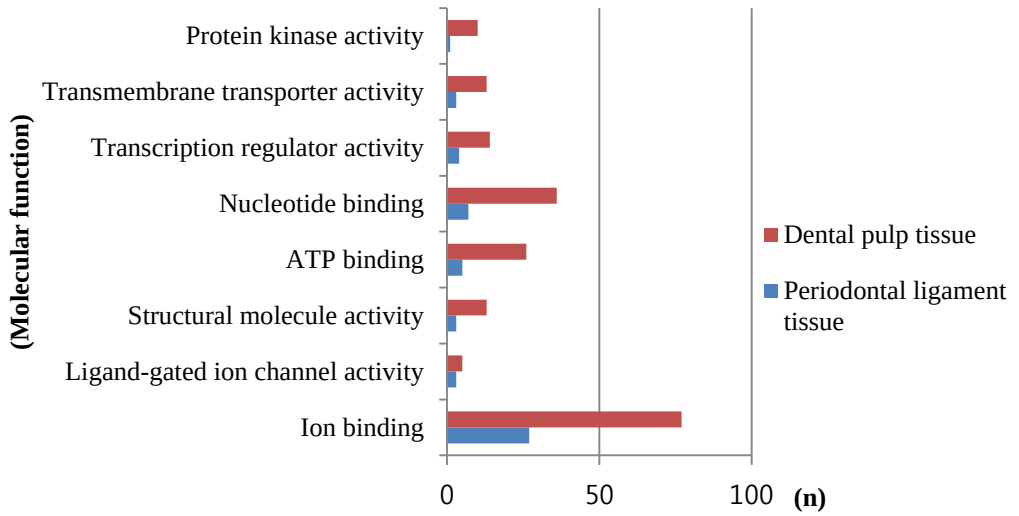

Figure 4. Main categories of genes expressed in the PDL and dental pulp tissues of the human permanent teeth on basis of their molecular functions (*F*-statistic $p<0.05$)

3. Quantitative RT-PCR

To verify the different gene expression levels obtained through cDNA microarray analysis, quantitative RT-PCR was performed. I selected the six genes, such as *COL12, MMP9, SPP1, CALB1, SPOCK3,* and *DSPP* for this verification procedure. The results of RT-PCR exhibited at least ninefold or greater increase in gene expression level to the other type of tissues (Table 4, 5). The results were consistent with those of cDNA microarray.

Table 4. mRNA expression ratios for the PDL/dental pulp tissue in permanent teeth

Gene	Relative Expression
COL12	44 ± 6
MMP9	18 ± 8
SPP1	9 ± 2

Table 5. mRNA expression ratios for the dental pulp/PDL tissue in permanent teeth

Gene	Relative Expression
CALB1	4,600 ± 270
SPOCK3	6,800 ± 270
DSPP	15,000 ± 2,500

4. Immunohistochemical staining

The following four proteins were the targets of the IHC study: *COL12A1, MMP9, SPP1, and CALB1* (Figure 5). *COL12A1* was strongly stained in PDL tissues, but not in pulp tissues. *MMP9* and *SPP1* were stained more strongly across the board in PDL tissues than pulp tissues, but in odontoblasts layer of the pulp tissues. *CALB1* was found to be located mainly in odontoblasts layer of pulp tissues. The results were consistent with those of the cDNA microarray analysis in the protein level.

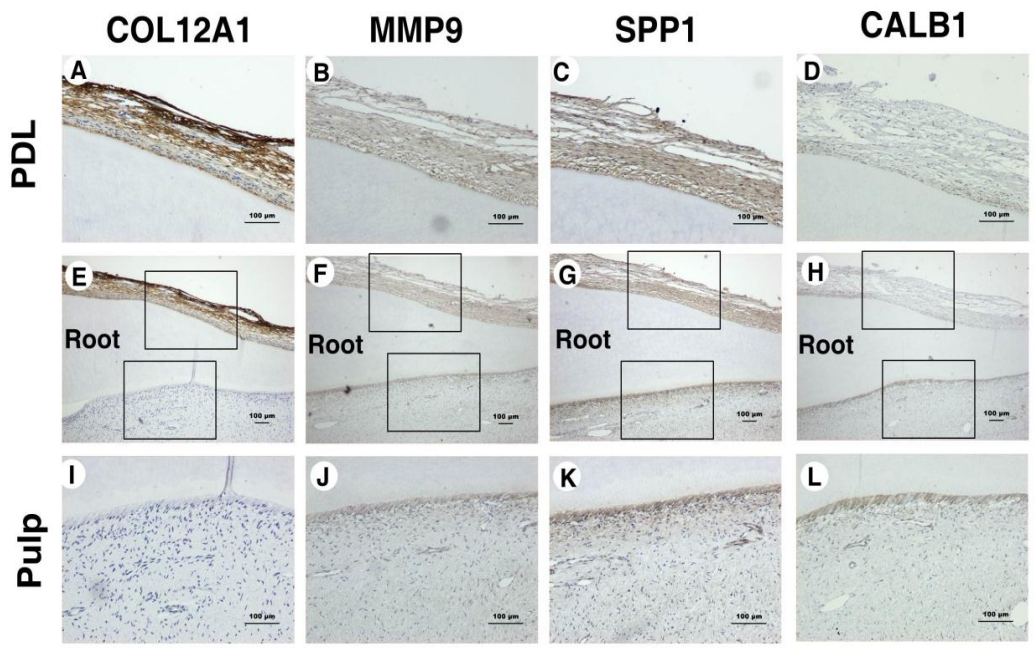

Figure 5. IHC staining of PDL and pulp tissue (A–L). (A, E) IHC staining for *COL12A1* in PDL and (E, I) pulp tissues. (B, F) IHC staining for *MMP9* in PDL and (F, J) pulp tissues. (C, G) IHC staining for *SPP1* in PDL and (G, K) pulp tissues. (D, H) IHC staining for *CALB1* in PDL and (H, L) pulp tissues. The micrographs in A–D and I–L are higher-magnification views of the areas outlined by squares in E–H. Scale bars: 100 μm in A-L

IV. Discussion

The PDL is a soft, fibrous specialized connective tissue which is present in the periodontal space, which is situated between the cementum of root of the tooth and bone forming the socket wall. It is derived embryologically from neural crest-derived ectomesenchymal cell of dental follicle that surrounds the developing tooth in its bony crypt (Ten Cate 1997) and plays an important role in supporting tooth function, maintaining homeostasis and repairing tissue damage cause by periodontal disease and mechanical trauma (Shimono et al. 2003). In several studies, specific expressed genes of the PDL were reported. The PDL cells in culture express alkaline phosphatase (Lekic and McCulloch 1996), bone sialoprotein 2 (Lallier et al. 2005), periostin (Han et al. 2002), osteopontin (Lekic and McCulloch 1996), and osteomodulin (Lallier et al. 2005), all being associated with mineralization. In addition, as an inhibitor of periodontal ligament mineralization, asporin (Yamada et al. 2007), matrix metalloproteinase (Kerrigan et al. 2000, Takahashi et al. 2003) were related with turnover of PDL matrix. It was reported that receptor activator nuclear factor κB ligand (RANKL), soluble macrophage colony stimulating factor (M-CSF) and osteoprotegerin (OPG) were associated with osteoclast differentiation (Kanzaki et al. 2001, Wada et al. 2001).

The dental pulp tissue is an unmineralized oral tissue composed of soft connective tissue, vascular, lymphatic and nervous element that occupies the central pulp cavity of each tooth. It is derived from neural crest-derived ectomesenchymal cell of dental papilla. The large number of undifferentiated mesenchymal cells facilitates the recruitment of newly differentiating cells to replace to others when they are lost, specifically odontoblasts. In recent days, it has been developed in a better understanding of the molecular events leading odontoblast differentiation and dentinogenesis.

Differentiation of odontoblast occurs during bell stage of tooth development and requires a cascade of signaling phenomena between ameloblasts and components of the ectomesenchyme of dental papilla (Hoffmann et al. 2002). The genes of specific

expressed in dentinogenesis were dentin phophoprotein, dentin sialoprotein, bone sialoprotein, osteocalcin, osteopontin and osteonectin (Butler 1998, Butler et al. 1997). The function of apoptosis and its regulation in odontoblasts remain unclear. But it was reported that the possible role of transforming growth factor beta 1 (TGF-β1) in the induction of apoptosis of odontoblasts (He et al. 2005).

Meanwhile, most studies were conducted by gene expression analysis using cultured cells of PDL and dental pulp *in vitro*, but there have been few *in vivo* studies about their gene expression analysis in tissue level. In the present study, I compared representative gene expression profiles between PDL and dental pulp tissues, using the cDNA microarray system. Several genes with different expression patterns in these tissues using microarray analysis were found. In addition, several genes with previously unknown strong expressions in PDL and dental pulp tissue were detected.

Genes related to collagen degradation, such as Matrix metalloproteinase 3 (*MMP3*), *MMP9*, *MMP13*, and with collagen synthesis, such as *FAP*, were more strongly expressed in PDL tissues than in dental pulp tissues of permanent teeth. It is thought that these strongly expressed genes associated with the collagen degradation and syntheses are related to PDL's high turnover rate.

MMP3, MMP9, MMP13 area member of the MMP family. Proteins of MMP family are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis, intracerebral hemorrhage (Wang and Tsirka 2005). The periodontal attachment of teeth is maintained by PDL fibroblasts that act to regulate and synthesize extracellular matrix molecules (collagens), cell surface receptors (integrins) and enzyme that degrade and remodel the extracellular matrix (MMPs). The MMPs are a family of proteolytic enzymes that mediate the degradation of extracellular matrix macromolecules (Hakki et al. 2009) and they contribute not only to extracellular matrix homeostasis, but also to pathologic or therapeutic situation. Concerning the pathologic situation (e. g., periodontal disease), several MMPs, such as MMP1, MMP3, MMP9, and MMP13, are induced in the resident fibroblasts and the recruited immune

cells (Kubota et al. 2008). In the therapeutic situation, it was reported that orthodontic tooth movement induced by mechanical force related with increase in MMP12 and MMP13 (Ziegler et al. 2010).

Fibroblast activation protein, alpha (FAP) known as seprase or 170 kDa melanoma membrane-bound gelatinase is a protein that in humans is encoded by the *FAP* gene. Fibroblasts play a major role in the production and turnover of extracellular matrix component, a process critical for wound healing and tissue remodeling (Elias 1992). This protein is thought to be involved in the control of fibroblast growth or epithelial-mesenchymal interactions during development, tissue repair, and epithelial carcinogenesis (Shi et al. 2012). FAP is widely known that it specifically induced in area of tissue injury or tumor stroma. Research of FAP in dentistry is rare, because of its lack of expression in normal tissue and induced expression in area of tissue remodeling and tumor stroma.

Genes related to bone development and remodeling, such as secreted phosophoprotein 1 (*SPP1*), bone morphogenetic protein 3 (*BMP3*), acid phosphatase 5, tartrate resistant (*ACP5)*, cathepsin K (*CTSK*), and parathyroid hormone-like hormone (*PTHLH*) were more strongly expressed in PDL tissues than in dental pulp tissues of permanent teeth. It is thought that these strongly expressed genes associated with bone development and remodeling are related to regulation of osteoclast activity in PDL.

SPP1, encoded by *SPP1* gene and widely known as osteopontin (OPN), has been implicated as an important factor in bone remodeling (Choi et al. 2008). Bone modeling and remodeling are integral processes involving multiple feedback loops between all cells involved (Blair et al. 2005, Boyle et al. 2003, Teitelbaum 2000, Wada et al. 2006). During bone remodeling, RANKL expressed by osteoblasts, coordinates local osteoclast development and resorption, which in turn stimulates bone synthesis by adjacent osteoblasts. Although osteoblasts are thought to be the major functional regulators of osteoclast activity, many other cell types also influence osteoclast activity, including PDL fibroblasts, which express RANKL in response to compressive forces *in vitro* and *in vivo* (Kanzaki et al. 2001, Oshiro et al. 2002, Yoshinaga et al. 2007). Another

important factor that mediates the osteoclast response to mechanical force is the extracellular matrix glycoprotein osteopontin (Terai et al. 1999). OPN is essential for the binding of integrin receptors, and has been found to be important for osteoclast chemotaxis and attachment to bone (Chellaiah and Hruska 2003, Faccio et al. 1998, Nomura and Takano-Yamamoto 2000, Xuan et al. 1995). Prior research has established that orthodontic forces increase the expression levels of both RANKL and OPN in the PDL and alveolar bone, respectively (Krishnan and Davidovitch 2006, Shiotani et al. 2001). There were another research that in the bone formation, several extracellular matrix macromolecules, including OPN, osteocalcin, bone sialoprotein that are expressed by differentiating osteogenic cells can identify discrete stage in the formation of bone *in vitro* (Stein et al. 1990). Specifically, research suggests that OPN plays a role in anchoring osteoclasts to the mineral matrix of bones (Reinholt et al. 1990). OPN is a multifunctional protein involved in bone turnover, osteoclastogenesis, and osteoblast function. In mouse model experiment, OPN was strongly expressed in the cellular cementum and alveoloar bone/PDL interface of mouse molar (Walker et al. 2008).

BMP3 is a protein in humans that is encoded by the *BMP3* gene. The protein encoded by this gene is a member of the transforming growth factor beta superfamily. BMP3 has been extensively investigated in bone formation. It was reported that it down-regulated bone mineralization and density and was an antagonist of osteogenic BMP (Daluiski et al. 2001). In the mouse's periodontium formation, it was localized on at the latest stage of periodontium formation by inhibiting the signaling provided by other BMPs (Kemoun et al. 2007).

ACP5, also known as tartrate resistant acid phosphatase (TRAP) and encoded by *ACP5*, is a glycosylated monomeric metalloenzyme expressed in mammals (Baumbach et al. 1991). It has a molecular weight of approximately 35kDa, and optimal activity in acidic conditions. In knockout studies, TRAP-/- mice exhibit mild osteopetrosis, associated with reduced osteoclast activity. In TRAP over-expressing transgenic mice, mild osteoporosis occurs along with increased osteoblast activity and bone synthesis (Angel et al. 2000). Under normal circumstances, TRAP is highly expressed by

osteoclasts, activated macrophages, neurons. It has been reported that OPN and bone sialoprotein are highly associated with TRAP substrate *in vitro*, which bind to osteoclasts when phosphorylated (Ek-Rylander et al. 1994). Upon partial dephosphorylation, both OPN and bone sialoprotein are incapable of binding to osteoclasts, which resulted in osteoclast migration.

CTSK is an enzyme that in humans is encoded by the *CTSK* gene. This protein, which is a member of the peptidase C1 protein family, is expressed predominantly in osteoclasts. Cathepsins are proteolytic enzymes that are involved in intracellular proteolysis (Mogi and Otogoto 2007). CTSK has an extremely powerful effect on collagen and gelatin degradation (Drake et al. 1996). High levels of CTSK are detected in patients with bone-destructive disease. Bone destruction resulting from periodontal disease is a process in which osteoclasts take part and is characterized by the resolution of inorganic minerals and proteolytic degradation of organic matrix, mainly type 1 collagen (Ohba et al. 2000). Periodontal studies analyzing CTSK levels in gingival crevicular fluid have demonstrated that the concentration of CTSK increased with degradation of bone matrix molecules (Mogi and Otogoto 2007). Based on these features, CTSK has been suggested as a good marker for osteoclast activity (Okaji et al. 2003).

PTHLH, encoded by *PTHLH*, is a member of the parathyroid hormone family. It regulates endochondral bone development and epithelial-mesenchymal interaction during formation of the mammary glands and teeth. Fresh PDL express higher quantities of PTHLH, which is involved in calcium and phosphate homeostasis and bone development than do PDL cell in culture (Gensure et al. 2005, Provot and Schipani 2005). PTHLH stimulates osteoclastogenesis by inducing tartrate-resistant acidic phosphatase in PDL cells, while increasing the receptor activator of the NF-κB ligand and decreasing osteoprotegerin levels (Fukushima et al. 2005). PTHLH represses bone sialoprotein, osteocalcin, and the mineralization in PDL cells and, potentially, in cementoblast (Berry et al. 2003, Ouyang et al. 2000). Thus, the PDL may use PTHLH to regulate the remodeling of associated mineral tissues.

In the dental pulp tissues of permanent teeth, genes associated with calcium ion such as those that encode Calbindin 1 (*CALB1*), cadherin type 2 (*CDH12*), and scinderin (*SCIN*) were more highly expressed.

Calbindin 1, encoded by *CALB1*, is intracellular, soluble, vitamin-D-dependent calcium binding protein and a member of the troponin C superfamily (Wasserman and Taylor 1966). Two classes of calbindins have been reported: a 28-kDa protein (calbindin 1) and a protein of 8–10 kDa (calbindin 2). The active transport of calcium across cells holds widespread importance in medicine and biology. Operating in many places (*e.g.* gut, kidney, placenta, teeth, bones, oviduct, lung, inner ear), active transport is used to control the amount of calcium in body fluids and so impacts on nutrition, biomineralization, fertility, respiration, and hearing. The phenomenon that calcium is ferried through cytosol by mobile calcium-binding proteins, such as calbindins, remains widely accepted (Peng et al. 2003). Active calcium transport is crucial for normal production of tooth enamel, and it appeared that the calbindin ferry model would apply here too. Enamel, the most highly calcified of all tissues, is formed in two stages (secretion, maturation) involving low and high rates of calcium transport, respectively. Calbindin 1 is expressed abundantly in enamel-forming cells in a vitamin D-dependent fashion, and calbindin 2 is also expressed at trace level (Berdal et al. 1996). Also, calbindin 1 is thought to be associated with the transportation and accumulation of intracellular calcium ion in odontoblasts. Therefore, calbindin 1 in odontoblasts probably plays an important role in dentin formation (Magloire et al. 1988).

Cadherin-12 is a protein that in humans is encoded by the *CDH12* gene. This gene mediates calcium-dependent cell-cell adhesion. This particular cadherin appears to be expressed specifically in the brain and its temporal pattern of expression would be consistent with a role during a critical period of neuronal development, perhaps specifically during synaptogenesis (Selig et al. 1995). Cell adhesion molecules are classified into four protein families: the cadherins, the integrins, the selectins, and the immunoglobulin superfamily proteins. Cadherins make up a large superfamily that can be divided into classical cadherins, desmosomal cadherins, protocadherins, seven transmembrane

cadherins, and FAT-like cadherins. E-cadherin (epithelial cadherin), N-cadherin (neuronal cadherin), and P-cadherin (placental cadherin) represent the most well-characterized classical cadherins and which were the first cadherins to be described (Takeichi 1988). Although their names imply tissue specificity, the accumulated knowledge shows that the expression of the different cadherins is not completely tissue-specific. The expression of E-cadherin and N-cadherin during embryonic tooth morphogenesis and their down-regulation in adult dental tissues indicate that these cadherins have important roles in the development and shaping of the dental organ. Both N-cadherin and E-cadherin proteins were detected in embryonic dental tissues and noticeable differences were observed in the expression patterns of the two cadherins. E-cadherin was initially expressed in the dental epithelium at the cap stage, and was down-regulated when ameloblast differentiation started at the bell stage. N-cadherin showed an inverse gradient compared with E-cadherin and was up-regulated in differentiated epithelial cells (Heymann et al. 2002).

Scinderin is a protein that in humans is encoded by the *SCIN* gene. Scinderin is an actin severing protein discovered in Dr. Trifaro's laboratory at the University of Ottawa, Canada. Secretory tissues are rich in scinderin. In these tissues, scinderin, a calcium dependent protein, regulates cortical actin networks. Normally secretory vesicles are excluded from release sites on the plasma membrane by the presence of a cortical actin filament network. During cell stimulation, calcium channels open allowing calcium ions to enter to secretory cell. Increase in intracellualr calcium activates scinderin with the actin filament severing and local dissociation of actin filament networks. This allows the movement of secretory vesicles to release sites on the plasma membrane.

In the dental pulp tissues of permanent teeth, genes associated with mineralization and formation of enamel or dentin such as those that encode secreted protein acidic and rich in cystein (SPARC)/osteonectin 3 (*SPOCK3*), phosphate regulating endopeptidase homolog, X-linked (*PHEX*), ameloblastin (*AMBN*), dentin sialophosphoprotein (*DSPP*) were more highly expressed.

Osteonectin, also known as SPARC is a protein that in humans is encoded by the *SPOCK3* gene. SPARC is a phosphorylated glycoprotein that is associated with

development, tissue remodeling, and repair (Yan and Sage 1999). It has recently been shown that odontoblasts, which are the only dental pulp cells expressing SPARC, have an increased expression of SPARC in the initial stage of tertiary dentin formation (Itota et al. 2001, Reichert et al. 1992). Although the function of SPARC in dentin formation remains to be determined, it has been suggested that odontoblasts release SPARC to stimulate the proliferation of a fraction of pulp cells to replace those injured cells by dental caries or by cavity preparation (Shiba et al. 2001). SPARC may also regulate production of the extracellular matrix and MMP, which might be involved in the modulation of matrix for dentinogenesis (Francki et al. 1999, Tremble et al. 1993).

Phosphate-regulating gene with homologies to endopeptidase, on the X-chromosome (PHEX) protein is an enzyme that in humans is encoded by the *PHEX* gene (Grieff et al. 1997). This gene contains 18 exons and is located on the X chromosome. This protein is a transmembrane endopeptidase that belongs to the type II integral membrane zinc-dependent endopeptidase family. The protein is thought to be involved in bone and dentin mineralization and renal phosphate reabsorption. To assess a functional role of PHEX, several investigators have tried to determine its tissue and cell distribution in humans and the mouse. PHEX mRNA was detected in mouse bones and teeth (Du et al. 1996) and in human fetal bone and lung as well as in the human adult ovary (Grieff et al. 1997). The expression of the *PHEX* gene was studied *in vivo* on human dental pulp by *in situ* hybridization. This results show that this gene is expressed by human odontoblasts aligned at the margin of the dental pulp, whereas no expression is detectable in the dental pulp cells (Bleicher et al. 2001). Moreover, PHEX is up-regulated during odontoblast development (Buchaille et al. 2000). Analysis of these data suggests that PHEX may play an important role in the mineralization process, and on the basis of its homology to endopeptidases, PHEX may activate or degrade a factor involved in the autocrine or paracrine regulation of bone and tooth mineralization.

Ameloblastin (AMBN), also known as amelin and encoded by *AMBN* gene, is a specific protein found in tooth enamel. Although less than 5% of enamel consists of protein, ameloblastin comprises 5%-10% of all enamel protein. This protein is formed by

ameloblasts during the early secretory to late maturation stages of amelogenesis. Although not completely understood, the function of ameloblastin is believed to be in controlling the elongation of enamel crystals and generally directing enamel mineralization during tooth development. *AMBN* gene expression has previously mainly been associated with odontogenesis and enamel formation. In recent studies, using amelogenin in the form of enamel matrix derivative, reparative dentin formation was associated with a sequential expression of dentin-specific factors in the wounded pulp in porcine teeth (Nakamura et al. 2001, Nakamura et al. 2002). The indication that AMBN can enhance reparative dentin formation suggests that this protein plays an important role during pulpal healing and links it firmly to odontoblast differentiation. It might have a potential clinical use in dentistry.

Dentin sialophosphoprotein, also known as DSPP and encoded by *DSPP* gene, is a human gene that encodes two principal proteins of the dentin extracellular matrix of the tooth. The predentin is secreted by odontoblasts and cleaved into dentin sialoprotein (DSP) and dentin phosphoprotein (DPP). A number of immunohistochemical studies using anti-DSP antibodies showed that DSP is localized in odontoblasts, predentin, and dentin (Baba et al. 2004, Butler et al. 1992). Based on the previous studies, DSPP is cleaved in the cells, and then DSP is secreted in the predentin, whereas DPP is secreted at the mineralization front and retained in the mineralized dentin. At the mineralization front, DPP binds to collagen fibrils and assumes a structure promoting the formation of initial apatite crystals. As the mineralization process proceeds and predentin is converted to dentin, these mineral crystals grow in an oriented fashion. DPP and other proteins bind to the growing hydroxyapatite faces and inhibit or slow crystal growth, which influences the size and shape of the apatite crystals (Prasad et al. 2010). The functions of DSP are unclear. But, a recent in vivo study indicates that DSP may be involved in the initiation of dentin mineralization but not in the maturation of this tissue (Boskey et al. 2000). It is thought that these strongly expressed genes (*CALB1, CDH12, SPOCK3, FHEX, AMBN,* and *DSPP***)** associated with calcium ion and odontoblasts are related to regulation of mineralization and formation of hard tissue.

The translation of such high-throughput gene-expression data into meaningful biological information and identification of associated signal pathways is a challenge to biologists. To achieve this, I chose to use the DAVID web-based tool. The identified genes were classified based on the information of gene function in the GO grouping and KEGG Pathway database. I grouped the gene for the biologic processes and molecular function of the genes. In broad outlines, the GO grouping results were that gene expressions of permanent dental pulp were more abundant than those of permanent PDL in same function. Especially, there were notable differences of biologic processes in regulation of cell adhesion, neurological system process, signal transduction, and ion transport, and of molecular functions in nucleotide binding, ATP binding, and ion binding (Figure 3, 4). This result means that dental pulp tissue is more specialized part of permanent teeth expressed by more various genes than PDL tissue.

cDNA microarray results are verified by confirmatory quantitative RT-PCR analyses. I selected the six genes of all of microarray data for quantitative RT-PCR. I chose *COL12*, *MMP9*, and *SPP1* for verification of up-regulation gene in permanent PDL tissues, and *CALB1*, *SPOCK3*, and *DSPP* in permanent dental pulp tissues. The results of RT-PCR exhibited at least ninefold or greater increase in gene expression level to the other type of tissues (Table 4, 5). The results were consistent with those of cDNA microarray.

To better understand location of cellular origin of the differentially expressed genes identified by our microarray analyses, IHC staining analysis was also used. *COL12A1* was strongly stained in PDL tissues, but not in pulp tissues. *MMP9* and *SPP1* were stained more strongly across the board in PDL tissues than pulp tissues, but in odontoblasts layer of the pulp tissues. This means that MMP9 and OPN expressed by odontoblasts in normal pulp. It is coincided with previous research shown that MMP9 may play an important role in the pathogenesis in pulpal inflammation (Gusman et al. 2002) and OPN synthesized by odontoblasts is associated with the initial sites of calcification within mantle dentin (Trowbridge 2003). *CALB1* was found to be located mainly in odontoblasts layer of pulp tissues. The results were consistent with those of the cDNA microarray analysis in the protein level (Figure 5).

The critical point of this research is that in obtaining the dental pulp and PDL tissues of permanent teeth, RNA composed with various kinds of cells could be collected. The further study for the cellular originality of expressed various genes were needed. And the biological function and mechanism of newly introduced gene in dental pulp and PDL of the human permanent teeth were necessary for future study in developing clinically treatment and tissue engineering.

V. Conclusion

This study was conducted to identify the gene-expression profiles and their molecular biological differences of PDL and dental pulp tissues from the human permanent teeth using cDNA microarray analysis, quantitative RT-PCR, and immunohistochemical stain. According to the results, the main findings are as follows.

1. The cDNA Microarray analysis identified 347 genes with a quadruple or greater difference in expression level between PDL and dental pulp tissues of the permanent teeth, 83 and 264 of which were more plentiful in PDL and dental pulp tissues, respectively.
2. Genes associated with collagen degradation such as *MMP3, MMP9,* and *MMP13* and with collagen synthesis such as *FAP*, and with bone development and remodeling such as *SPP1, BMP3, ACP5, CTSK,* and *PTHLH* were more strongly expressed in PDL tissues of permanent teeth. They are clinically related to PDL's functions of external force absorption and tooth supporting. .
3. In dental pulp tissues of permanent teeth, genes associated with calcium ion such as *CALB1, CDH12,* and *SCIN* and with mineralization and formation of enamel or dentin such as *SPOCK3, PHEX, AMBN*, and *DSPP* were more strongly expressed. They are clinically related to dental pulp's functions of secondary and tertiary dentin formation.
4. The quantitative RT-PCR analysis which was conducted for 6 randomly selected genes was consistent with the results of the cDNA microarray assay.
5. The immunohistochemical staining analysis showed that *COL12A1, MMP9,* and *SPP1* was extensively expressed in permanent PDL tissues, but barely expressed in permanent dental pulp tissues. *CALB1* was extensively expressed in the permanent dental pulp tissues, but barely expressed in permanent PDL tissues. These results were also coincided with the cDNA microarray assay data.

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국문요약

사람 영구치에서 치주인대 및 치수 조직의 유전자 발현에 대한 비교 연구

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이 석 우

지도교수 : 김 성 오

영구치의 치주인대 및 치수 조직은 발생학적인 과정, 조직학적 특성 및 기능 등에서 분명한 차이점을 가지고 있으며, 이러한 차이는 영구치 치주인대 조직 및 치수 조직을 이루는 세포가 다른 유전자 발현을 하고 있음을 짐작할 수 있다. 그 동안 영구치 치주인대와 치수의 세포 배양 차원 (*in vitro*)에서의 유전자 연구는 수행되어 왔으나, 기능하는 조직 차원 (*in vivo*)에서의 유전자 비교 연구는 거의 없었다. 본 연구는 사람 영구치에서 치주인대 및 치수 조직 내의 유전자 발현을 규명하고 각각의 분자생물학적 차이를 알아보기 위해서 영구치 치주인대 및 치수 조직을 이용하여 cDNA 미세배열 (cDNA microarray analysis) 분석과 역전사효소 중합효소 연쇄반응 (quantitative real time polymerase chain reaction) 분석과 면역조직화학염색법 (immunohisto-chemical analysis)을 시행하여 다음과 같은 결론을 얻었다.

1. cDNA 미세배열 분석 결과, 영구치 치주인대에서는 83 개의 유전자가, 영구치 치수에서는 264 개의 유전자가 4 배 이상 발현되었다.
2. 영구치 치주인대에서 matrix metallopeptidase 3 (*MMP3*), matrix metallopeptidase 9 (*MMP9*), matrix metallopeptidase 13 (*MMP13*)

등 “교원질 분해(collagen degradation)” 및 fibroblast activation protein, alpha (*FAP*) 등 “교원질 합성(collagen synthesis)” 과 연관된 유전자와 secreted phosphoprotein 1 (*SPP1*), bone morphogenetic protein 3 (*BMP3*), acid phosphatase 5, tartrate resistant (*ACP5*), cathepsin K (*CTSK*), parathyroid hormone−like hormone (*PTHLH*) 등 “골 형성 및 개조(bone development and remodeling)” 과 연관된 유전자들이 치수보다 높게 발현되었다.

3. 영구치 치수에서 calbindin 1 (*CALB1*), cadherin 12, type 2 (*CDH12*), scinderin (*SCIN*) 등 “칼슘 이온(calcium ion)” 과 연관된 유전자와 secreted protein acidic and rich in cysteine/osteonectin 3 (*SPOCK3*), phosphate regulating endopeptidase homolog, X−linked (*PHEX*), ameloblastin (*AMBN*), dentin sialophosphoprotein (*DSPP*) 등 “법랑질 또는 상아질의 광화 및 형성(mineralization and formation of enamel or dentin)” 과 연관된 유전자들이 치주인대보다 높게 발현되었다.
4. 위의 유전자들 중 6 개의 유전자를 선택하여 역전사효소 중합효소 연쇄반응 분석을 시행하였는데, 이는 cDNA 미세배열 분석 결과와 동일하였다.
5. 면역조직화학염색 분석 결과 collagen, type XII, alpha 1(*COL12A1*), *MMP9, SPP1* 은 영구치의 치주인대에서 넓게 발현되었고, 반면에 영구치의 치수에서는 거의 관찰되지 않았다. *CALB1* 은 영구치의 치수에서 넓게 발현되었고, 반면에 영구치의 치주인대에서는 거의 관찰되지 않았다.

핵심되는 말: 영구치, 치주인대 조직, 치수 조직, cDNA 미세배열, 역전사효소 중합효소 연쇄반응 분석, 면역조직화학염색법